# Relative Contributions of the Egg Layer and Egg Cordon to Pheromonal Attraction and the Induction of Mating and Egg-laying Behavior in *Aplysia*

SHERRY D. PAINTER, MARIA G. CHONG, MARY A. WONG, ANN GRAY, JEFFRY G. CORMIER, AND GREGG T. NAGLE

*The Marine Biomedical Institute and the Department of Anatomy and Neurosciences, University of Texas Medical Branch, Galveston, Texas 77550-2781*

**Abstract.** Many species of the opisthobranch mollusk *Aplysia* form breeding aggregations during the reproductive season. The aggregations contain both mating and egg-laying animals, and are associated with egg cordons. Although pheromones play a significant role in developing and maintaining the aggregations, little is known about the active factors. Behavioral studies have shown that egg-laying animals are more attractive than nonlaying animals, have shorter latencies to mating, and induce conspecifics to lay eggs. As a first step toward isolating and chemically characterizing the active factors, we examined the relative importance of the egg layer and egg cordon as sources of pheromonal activity in *Aplysia brasiliana*. T-maze experiments showed that both animal-derived and cordon-derived factors are attractive, and that the animal-derived factors are specifically associated with egg layers. Extracts of the atrial gland—an exocrine organ secreting into the oviduct—increased the attractiveness of nonlaying animals when placed in the surrounding seawater, suggesting that the "cordon-derived" aggregation pheromones may be products of the atrial gland. Mating studies showed that both animal-derived and cordon-derived factors induce mating, and that the animal-derived factors are associated with both egg layers and nonlayers. In contrast, neither animal-derived nor cordon-derived factors induced egg laying. Comparable results were obtained with either one or two animals in the chamber, suggesting that the accessibility of a potential mate did not influence the results. The lack of effect may result from the low-probability nature of egg-laying activity.

## Introduction

Pheromones appear to play an important role in coordinating reproductive activities in the marine opisthobranch mollusk *Aplysia*. Field studies of both *A. californica* (Kupfermann and Carew, 1974; Audesirk, 1979) and *A. fasciata* (Susswein *et al.*, 1983, 1984) indicate that these simultaneous hermaphrodites are solitary animals during most of the year. During the summer reproductive season, however, they move into breeding aggregations or "brothels." The aggregations are typically associated with recently deposited egg cordons, often laid one on top of another, and contain both mating and egg-laying animals. Most of the egg-laying animals simultaneously mate as females, even though mating does not cause reflex ovulation (*A. brasiliana*, Blankenship *et al.*, 1983), suggesting that egg laying may precede mating in the aggregations rather than result from it.

This sequence of activities is important because of the reproductive physiology of *Aplysia*. Four characteristics of the system are worth noting. First, *Aplysia* are non-self-fertilizing hermaphrodites that store large amounts of exogenous sperm for future use (*A. californica*, MacGinitie, 1934). Second, many species of *Aplysia* lay egg cordons containing from one to several million fertilized eggs (*e.g., A. californica*, MacGinitie, 1934; *A. depilans* and *A. fasciata*, Thompson and Bebbington, 1969; *A. dactylomela* and *A. juliana*, Switzer-Dunlap and Hadfield, 1977). In these species, a single bout of egg laying significantly reduces the amount of exogenous sperm stored, although the stores are not depleted. Third, animals lay

eggs regardless of whether sperm are available to fertilize them (*A. californica*, MacGinitie, 1934), suggesting that there is not a strong internal link between the amount of exogenous sperm stored and egg deposition. Finally, sperm are transferred between animals in an immature state, and cannot immediately fertilize the recipient's oocytes (*A. depilans, A. fasciata,* and *A. punctata,* Thompson and Bebbington, 1969). Mating as a female during, or shortly after, egg deposition thus ensures that mature and capacitated sperm will be available to fertilize oocytes during future egg-laying episodes.

Laboratory experiments suggest that the egg layer or its egg cordon are potential sources of pheromones that attract conspecifics and induce mating activity (*A. brasiliana*, Aspey and Blankenship, 1976; Jahan-Parwar, 1976; Painter *et al.*, 1989; *A. californica*, Jahan-Parwar, 1976; Audesirk, 1977); there is also evidence suggesting that they induce egg laying (*A. californica*, Audesirk, 1977) and may be responsible for the masses of egg cordons associated with the aggregations. Since mating animals are no more attractive than nonmating animals (*A. dactylomela,* Lederhendler *et al.*, 1977) and mating does not cause reflex ovulation (*A. brasiliana*, Blankenship *et al.*, 1983), the layer-derived or cordon-derived factors are likely to be responsible for developing and maintaining the breeding aggregations.

Although relatively little is known about pheromonal regulation of any component of *Aplysia* reproductive activity, a great deal is known about the neuroendocrine regulation of one component—egg laying. The bag cells, two clusters of neurosecretory cells located at the rostral margin of the abdominal ganglion (*A. californica,* Frazier *et al.*, 1967; *A. brasiliana,* Blankenship and Coggeshall, 1976) are part of the final common pathway leading to egg deposition. These normally quiescent cells periodically enter a prolonged phase of rapid and synchronous electrical activity (*A. californica*, Kupfermann and Kandel, 1970; *A. brasiliana,* Dudek and Blankenship, 1977) which releases several peptides into the connective tissue sheath surrounding the bag-cell clusters and abdominal ganglion (*A. californica,* Stuart *et al.,* 1980); this activity is consistently followed by egg laying (*A. brasiliana,* Dudek *et al.,* 1979). Most of the released peptides have been isolated and chemically characterized, including the ovulation-inducing egg-laying hormone (ELH) (*A. californica,* Chiu *et al.,* 1979; *A. brasiliana,* Nagle *et al.,* 1988a) and the autocrine alpha bag-cell peptide ($\alpha$BCP) (*A. californica,* Rothman *et al.,* 1983).

The bag-cell products that have been characterized to date are all encoded by the ELH gene, one member of a small family of structurally related genes that are expressed in a tissue-specific manner in *Aplysia* (*A. californica,* Scheller *et al.,* 1983; Mahon *et al.*, 1985). Each gene encodes a precursor protein containing a sequence homol-ogous to ELH and one or more sequences homologous to $\alpha$BCP. Although the gene sequences are highly conserved (*ca.* 90% identity), the resulting precursor proteins are processed into homologous but nonidentical sets of peptides with similar pharmacological properties but different physiological functions.

Several ELH-related genes are expressed in the atrial gland (*A. californica,* Scheller *et al.,* 1983; Mahon *et al.*, 1985), an exocrine organ secreting into the oviduct of *Aplysia* (*A. californica,* Arch *et al.,* 1980; Beard *et al.,* 1982; Painter *et al.,* 1985). Although extracts of this organ induce egg laying when injected into receptive animals (*A. californica,* Arch *et al.,* 1978), the exocrine nature of its secretory activity indicates that the atrial gland products do not function as hormones to induce egg laying. Recent behavioral studies suggest that the atrial gland products may function as sexual pheromones instead (Painter *et al.*, 1989; also see Susswein and Benny, 1985). Peptide products of a small family of structurally related genes may thus act as nonsynaptic neurotransmitters, neurohormones, and pheromones to regulate and coordinate both male and female reproductive activities.

The studies presented in this paper examine the relative contributions of the egg layer and its cordon to pheromonal attraction and to the pheromonal induction of both mating and egg-laying activity. They demonstrate that the egg cordon plays an important role in both attraction and induction of mating, and suggest that products of the atrial gland may contribute to these activities.

## Materials, Methods, and Results

### Animals

Specimens of *Aplysia brasiliana* (Rang), weighing from 100 to 400 g, were collected from South Padre Island, Texas, and were used in experiments between June and August, the normal reproductive season for this species. *Aplysia brasiliana* was selected as the experimental animal because it lays eggs and mates more frequently than *A. californica* (Pinsker and Parsons, 1985), and can be collected in large numbers from the south Texas coast during the reproductive season. The animals were housed in individual cages in one of five large aquaria containing recirculating artificial seawater (ASW; Instant Ocean, Aquarium Systems, Mentor, Ohio) at room temperature ($20 \pm 2°C$); the salinity ranged from 30 to 32 ppt. A 14:10 light:dark cycle was maintained, with the light period starting at 6 am; animals were fed dried laver in the late afternoon (4–6 pm) after experiments were completed.

Specimens of *Aplysia californica* (Cooper) were purchased from Alacrity Marine Biological Services (Redondo Beach, California) and were used as a source of atrial glands for extracts. They were maintained as described

above for *A. brasiliana*, except that the ASW was cooled to 14 ± 2°C.
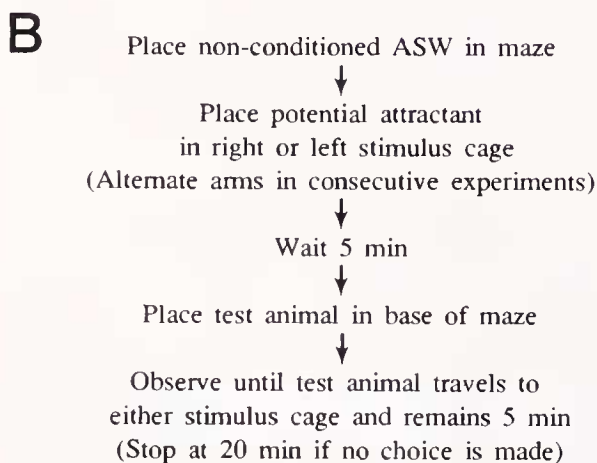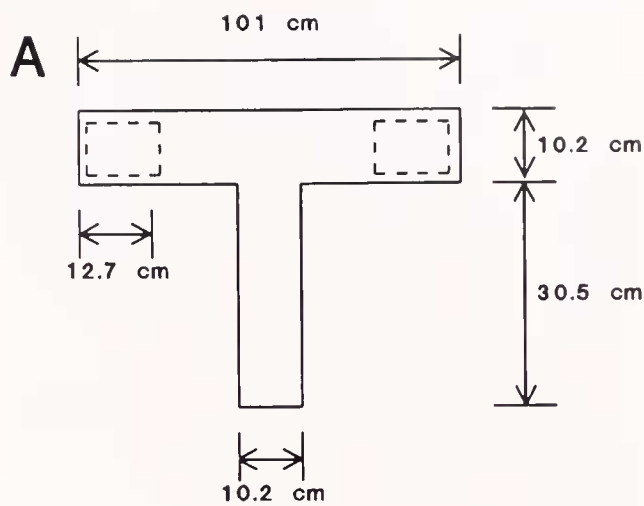
*Atrial gland extracts*

Sexually mature individuals of *Aplysia californica* were used as sources of tissue for extracts because the atrial gland in this species is large and well-defined; the homologous organ in *A. brasiliana* [the atrial gland-like epithelium (AG-LE)] is a thin band of secretory tissue that extends along the length of the oviduct and is difficult to recognize except in fixed or sectioned material (Painter *et al.*, 1985). Both organs secrete into the oviduct (Painter *et al.*, 1985), and there is evidence that their peptide products are highly conserved: (1) extracts of either organ induce egg laying when injected into receptive animals of either species (Painter *et al.*, 1985; also see Blankenship *et al.*, 1983); (2) secretory granules of either organ are specifically labeled by antisera raised against low molecular weight peptides isolated from the *A. californica* atrial gland (Painter *et al.*, 1985); and (3) restriction enzyme maps of the ELH-related genes in the two species suggest a high degree of sequence identity (Nambu and Scheller, 1986).

In most cases, the extracts were used as a tool to induce egg laying—0.1 ml of an ASW extract of the gland was injected through the foot into the hemocoel, and egg deposition began within approximately 30 min. Extracts were prepared by removing atrial glands (approximately 100 mg wet weight per gland) from 10 animals and homogenizing them in 10 ml of filtered ASW with a Brinkmann Polytron homogenizer (setting 4 for 1 min at 4°C). The homogenate was centrifuged at $1500 \times g$ for 30 min at 4°C, the supernatant removed, and aliquots frozen at −20°C until use.

Since previous studies have shown that acidic extracts of the *A. californica* atrial gland induce *A. brasiliana* to mate (Painter *et al.*, 1989), acidic extracts of the *A. californica* atrial gland were directly assayed for pheromonal attractiveness in *A. brasiliana*. In this case, atrial glands were removed from 10 animals and homogenized in 15 ml of 1 *M* acetic acid containing 20 m*M* HCl with a Brinkmann Polytron homogenizer (setting 4 for 1 min at 4°C). The homogenate was centrifuged at 48,000 × g for 20 min at 4°C, the supernatant removed, and the pellet rehomogenized in 5 ml of the acidic medium. After centrifugation, the supernatants were combined, distributed into 20 aliquots, lyophilized, and stored at −20°C until use. Each lyophilized aliquot was resuspended in 1 ml of ASW immediately before addition to the maze, and was equivalent to approximately 50% of the material in one gland.

*Pheromonal attraction*

*Apparatus.* A T-maze (Fig. 1A) was constructed of clear Plexiglas (0.62 cm thick) and sealed with clear aquarium



**A**

101 cm

10.2 cm

12.7 cm

30.5 cm

10.2 cm

**B**

Place non-conditioned ASW in maze
↓
Place potential attractant
in right or left stimulus cage
(Alternate arms in consecutive experiments)
↓
Wait 5 min
↓
Place test animal in base of maze
↓
Observe until test animal travels to
either stimulus cage and remains 5 min
(Stop at 20 min if no choice is made)

RESPONSES:
(+):   Travels to cage containing stimulus
(-):   Travels to opposite cage
(NC):  No choice; does neither

**Figure 1.** (A) Schematic diagram of the T-maze with removable stimulus cages (dashed outlines) in place. T-maze depth: 10.2 cm. (B) Flow diagram of the experimental protocol.

cement; it was cured for several days in ASW before use. Experiments were performed in a room adjacent to the aquarium facility with overhead fluorescent lighting; the maze was positioned so that the lighting was uniform throughout the apparatus.

*Experimental protocol and statistical analyses.* In a typical experiment (Fig. 1B), 6 l of aerated ASW that had not previously contacted an *Aplysia* ("non-conditioned ASW") was placed in a cleaned and air-dried maze. A potential attractant, usually consisting of a "stimulus" animal with or without an egg cordon or extract, was

placed in a perforated plastic cage in one arm. A non-treated sexually mature "test" animal was placed in the base of the maze 5 min later. Both the stimulus and test animals were briefly rinsed in non-conditioned ASW before being introduced into the maze. A response was considered to be positive if the test animal travelled to the stimulus within 20 min and remained in contact with the stimulus cage for 5 min, negative if it travelled to the opposite arm and remained for 5 min, and no choice if it did neither. Animals were choosing between a stimulus and no stimulus in these experiments, rather than between two qualitatively or quantitatively different stimuli. Fifteen experiments were performed for each potential attractant, and the attractant was alternated between arms in consecutive experiments. Statistical significance was assessed by $\chi^2$ analyses.

*Animals.* A total of 183 *Aplysia brasiliana* individuals were used in these studies. All were sexually mature—they laid eggs spontaneously or in response to injections of atrial gland extract—and all had been observed mating before they were separated into individual cages. Four criteria were used to select animals for each experiment: (1) the animal must not have laid eggs during the preceding 24 h; (2) the animal must not have participated in a behavioral experiment during the preceding 24 h; (3) the animal must not have been a test animal for the stimulus being studied; and (4) the test and stimulus animals must have been housed in the same aquarium. Egg layers were animals that had been injected with atrial gland extract to induce egg laying and, unless otherwise specified, had completed laying eggs within the preceding 30 min; nonlayers were animals that had not laid eggs during the preceding 24 h. Only nonlayers were used as test animals; both egg layers and nonlayers were used as stimulus animals.

*Results.* To assess directional bias and chance levels of attraction in the maze, 15 experiments were performed in which no stimulus was placed in either arm. Four of the animals (26.7%) moved to the left arm and remained, three (20%) moved to the right arm and remained, while eight (53.3%) did neither (Fig. 2), demonstrating that there is no directional bias in the maze and establishing chance levels of attraction at 3–4 animals (20–26.7%).

A similar level of attraction (3 animals; 20%) and pattern of responses was observed when a nonlaying conspecific without an egg cordon was the stimulus (Fig. 2). The two sets of responses were not significantly different $[\chi^2(2) = 0.14; 0.90 < P < 0.95]$, demonstrating that nonlayers without egg cordons are not attractive to *Aplysia brasiliana*. A higher level of attraction (12 animals; 80%) and lower level of no-choice responses (1 animal; 6.7%) was observed when the stimulus was a conspecific that was actively laying eggs (Fig. 2). This pattern of responses was significantly different from that to nonlaying animals
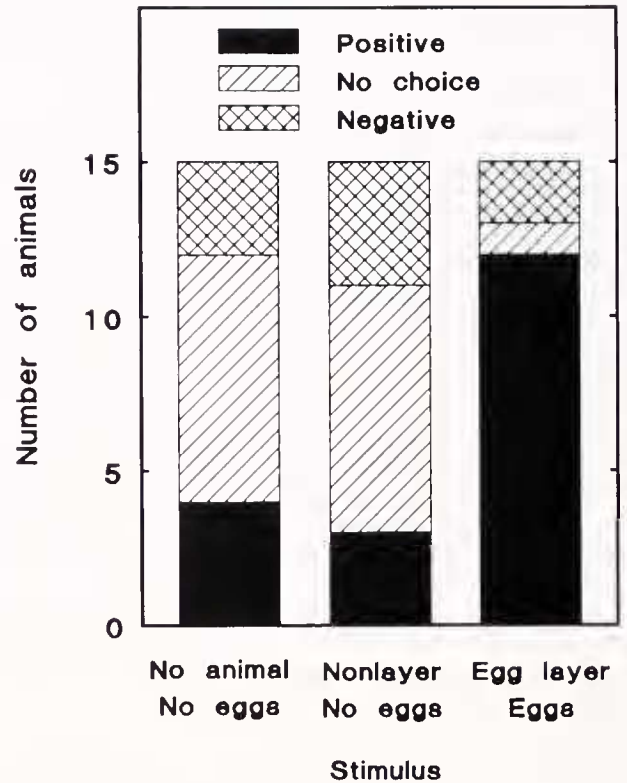


**Figure 2.** There is no directional bias in the T-maze, as indicated by the distribution of responses to the no-stimulus control (no animal, no eggs): approximately equal numbers of animals travelled to the left and right arms of the maze. (Note: for this quantification, movement to the left was defined as a positive response and movement to the right a negative response.) *Aplysia brasiliana* is not attracted to nonlaying animals without egg cordons: the response pattern did not differ significantly from that observed with the no-stimulus control $[\chi^2(2) = 0.14; 0.90 < P < 0.95]$. *A. brasiliana* are attracted to egg-laying conspecifics with egg cordons, however: the level of attraction and pattern of responses differed significantly from those observed when a nonlayer without eggs was the stimulus $[\chi^2(2) = 34.12; P < 0.005]$. This bar graph is based on 15 no-stimulus control experiments and 30 single-arm experiments, 15 each for "nonlayer, no eggs" and "egg layer, eggs"; in the single-arm experiments, animals were choosing between a stimulus in one arm and no stimulus in the other.

without egg cordons $[\chi^2(2) = 34.12; P < 0.005]$, demonstrating that egg-laying animals are attractive.

Because the "egg-laying animal" stimulus has two components—the egg layer and its egg cordon—subsequent experiments focused on their relative contributions to pheromonal attraction. More animals were attracted to egg layers without egg cordons than had been attracted to nonlayers without egg cordons, and fewer made no choice (Fig. 3); the difference in response patterns was statistically significant $[\chi^2(2) = 15.38; P < 0.005]$, demonstrating that the egg layer is a source of aggregation pheromones. Identical results were obtained when a nonlayer with a recently deposited egg cordon was used as the stimulus (Fig. 3), indicating that the egg cordon is also
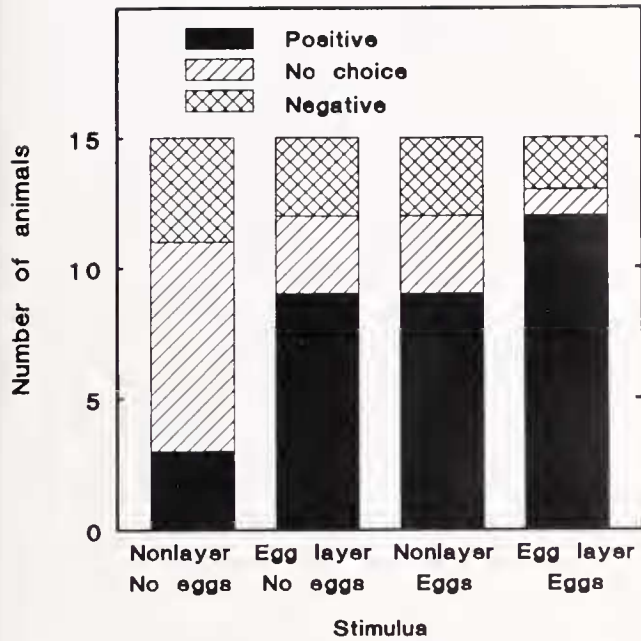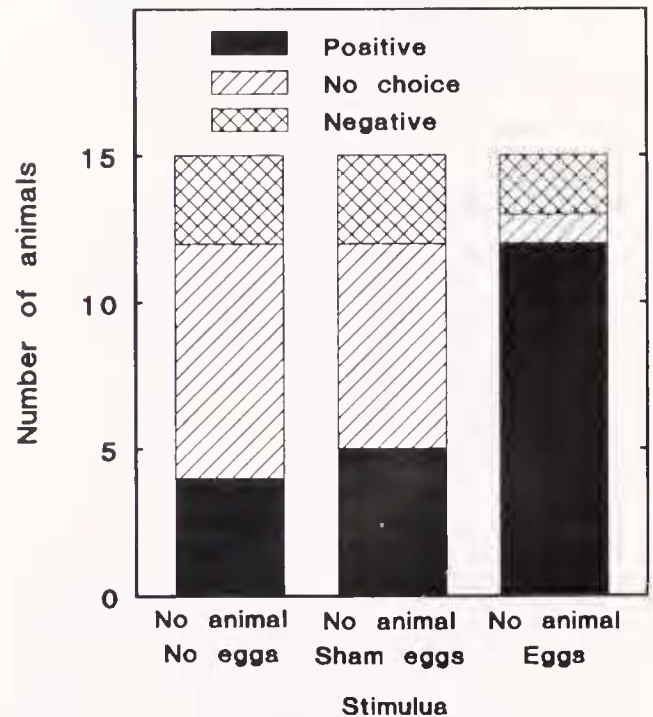
**Figure 3.** Egg layers are attractive to *Aplysia brasiliana:* a larger number of animals was attracted to an egg layer without eggs than was attracted to a nonlayer without eggs, and the patterns of responses to the two stimuli are significantly different [$x^2(2) = 15.38; P < 0.005$]. Egg cordons are also attractive to *A. brasiliana:* a larger number of animals was attracted to a nonlayer with eggs than was attracted to a nonlayer without eggs, and the response patterns are significantly different [$x^2(2) = 15.38; P < 0.005$]. The effects of egg layers and egg cordons are not additive at the concentrations tested: the response patterns for a nonlayer with eggs and an egg layer without eggs are identical, and do not differ significantly from the pattern obtained for an egg layer with eggs [$x^2(2) = 2.83; 0.25 < P < 0.50$]. This bar graph is based on 60 single-arm experiments, 15 per stimulus; in each experiment, animals were choosing between a stimulus in one arm and no stimulus in the other.

a source of pheromonal activity. Neither pattern differed significantly from that obtained for an egg-laying animal with an egg cordon [$x^2(2) = 2.83; 0.10 < P < 0.25$], demonstrating that the effects of the layer-derived and cordon-derived factors are not additive at the concentrations tested.

Subsequent experiments examined whether animal-derived factors are required for the attractiveness of the egg cordon and whether the attraction is visually mediated. Two series of experiments were performed. In the first, an egg cordon without any animal served as the stimulus. The level of attraction and pattern of responses were identical to those obtained using an egg layer and its cordon as the stimulus (Figs. 3, 4), demonstrating that egg cordons are sufficient to attract conspecifics. In the second series of experiments, a "sham" cordon (a tangled mass of silastic tubing; vol = 2 ml) served as the stimulus. The level of attraction and pattern of responses differed significantly from those observed with the egg cordon [$x^2(2) = 15.27; P < 0.005$], but did not differ from those observed

in no-stimulus control experiments [Fig. 4; $x^2(2) = 1.70; 0.25 < P < 0.50$]. The differential responses to egg cordons and sham cordons suggests that the attraction is chemically rather than visually mediated. The results of these two series of experiments, in conjunction with those reported above, demonstrate that both egg layers and egg cordons are sufficient to attract conspecifics, but that neither is uniquely required.

As a first step toward identifying tissue sources of the cordon-derived aggregation pheromones, acidic extracts of the atrial gland (equivalent to 50% of the material in one gland) were assayed for the ability to increase the attractiveness of a nonlaying animal when placed in the surrounding ASW. A higher level of attraction to nonlaying animals and lower level of no-choice responses was observed when the extract was present (Fig. 5); the dif-



**Figure 4.** Animals are not required for an egg cordon to be attractive to *Aplysia brasiliana:* a larger number of animals was attracted to an egg cordon without an animal than was attracted to a sham cordon without an animal, and the response pattern was significantly different [$x^2(2) = 15.27; P < 0.005$]. The sham cordon was a tangled mass of silastic tubing, 2 ml in volume. The attractiveness of an egg cordon is not visually mediated: the pattern of responses to a sham cordon without an animal did not differ significantly from the no-stimulus control (no animals, no eggs) [$x^2(2) = 1.70; 0.25 < P < 0.50$]. (Note: for the no-stimulus control, movement to the left was defined as a positive response and movement to the right a negative response.) This bar graph is based on 15 no-stimulus control experiments and 30 single-arm experiments, 15 each for "no animal, sham eggs" and "no animal, eggs"; in the single-arm experiments, animals were choosing between a stimulus in one arm and no stimulus in the other.
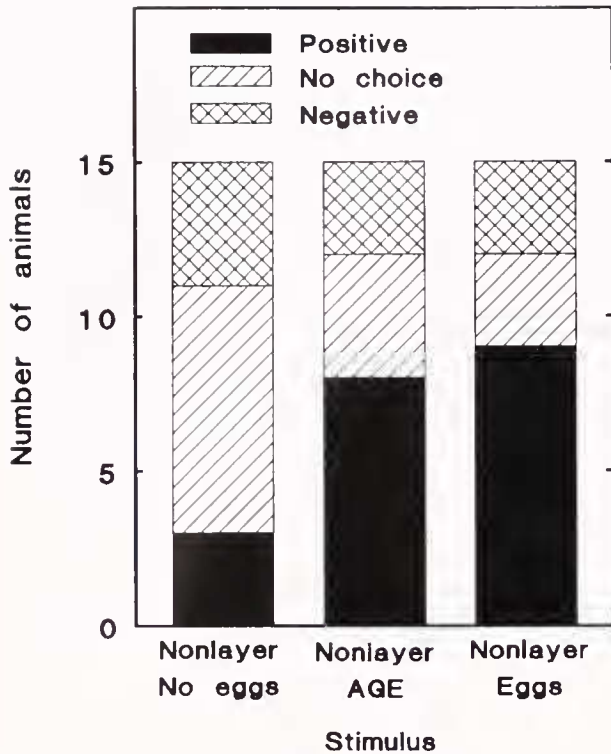
**Figure 5.** Secretory products of the *Aplysia californica* atrial gland (or *A. brasiliana* AG-LE) may contribute to the attractiveness of an egg cordon. The number of animals attracted to a nonlayer was increased when an extract of the *A. californica* atrial gland (AGE) was placed in the surrounding ASW. The pattern of responses differed significantly from that observed for a nonlaying animal without eggs [$x^2(2) = 10.58$; $P < 0.01$], but did not differ significantly from that for a nonlaying animal with eggs [$x^2(2) = 0.44$; $0.90 < P < 0.95$]. This bar graph is based on 45 single-arm experiments, 15 per stimulus; in each experiment, animals were choosing between a stimulus in one arm and no stimulus in the other.

ference in response patterns was significant [$x^2(2) = 10.58$; $P < 0.01$]. Interestingly, the level of attraction and pattern of responses did not differ significantly from those obtained when a recently deposited conspecific egg cordon was placed in the same location [Fig. 5; $x^2(2) = 0.44$; $0.75 < P < 0.90$], suggesting that products of the *A. californica* atrial gland (or *A. brasiliana* AG-LE) might significantly contribute to the attractiveness of an egg cordon.
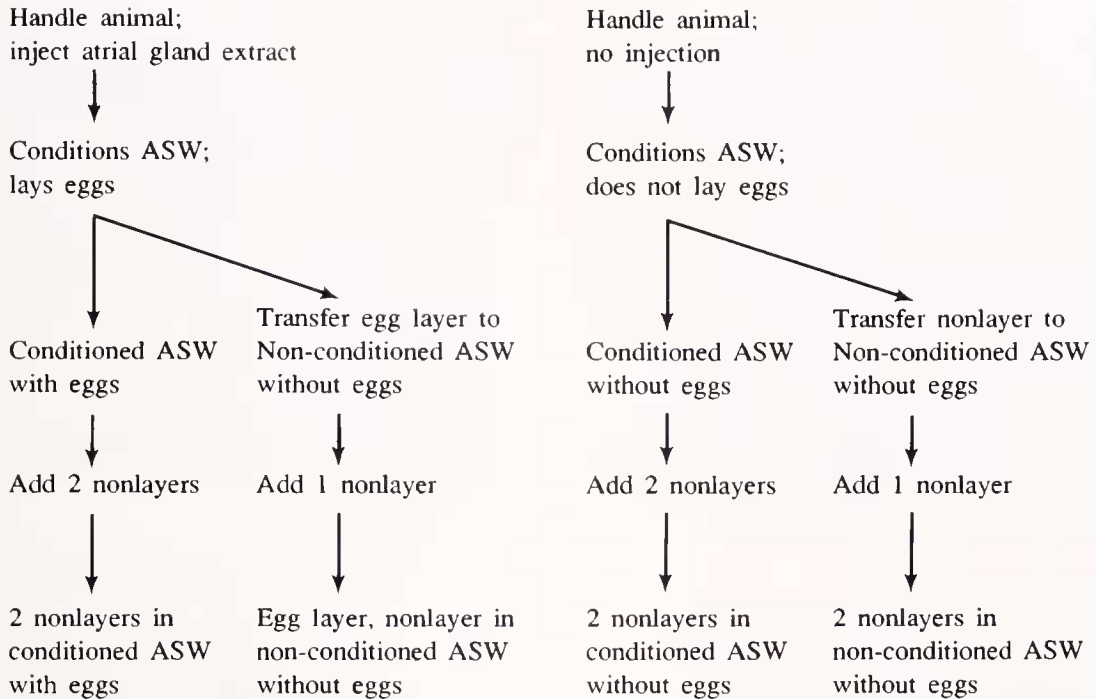
### Induction of mating activity

*Animals.* A pool of 205 sexually mature *Aplysia brasiliana* individuals was used in these studies. Small plastic fish tags (11 mm in diameter; Howitt Plastics, Molalla, Oregon) were sutured to the caudal region of the right parapodium so that individuals could be identified. Four criteria were used to select animals for each experiment: (1) the animal must not have laid eggs during the preceding 24 h; (2) the animal must not have participated in a

behavioral experiment during the preceding 24 h; (3) the animal must not have been tested with the stimulus; and (4) all of the animals in an experiment must have been housed in the same aquarium. Once selected, the animals were randomly assigned to treatments. All experiments were begun between 9 and 10 am, because there is evidence of a circadian rhythm in *Aplysia* mating behavior (*A. fasciata*, Susswein *et al.*, 1983, 1984).

*Relative contributions of the egg layer and egg cordon: experimental protocol and statistical analyses.* Eight animals were used in each experiment (Fig. 6). One animal was injected with 0.1 ml of atrial gland extract and placed in a 4-l plastic beaker containing 3 l of aerated non-conditioned ASW; this treatment induced egg laying, usually within 30 min, and the egg layer conditioned the ASW in the beaker. A second animal was handled and placed in an identical beaker; this treatment did not induce egg laying, but the nonlayer conditioned the ASW. When the injected animal finished laying eggs (70.3 ± 4.1 min after injection; mean ± S.E.M.), it was removed, rinsed in fresh non-conditioned ASW and transferred to a third beaker; the handled animal was treated in the same way and transferred to a fourth beaker. Nontreated animals were then distributed among the four beakers so that each contained two animals. The resulting experimental conditions are: (1) two nonlayers in animal-conditioned ASW with an egg cordon; (2) two nonlayers in animal-conditioned ASW without an egg cordon; (3) one egg layer and one nonlayer in non-conditioned ASW without an egg cordon; and (4) two nonlayers in non-conditioned ASW without an egg cordon.

The reproductive activity of each individual was assessed at 10-min intervals for 270 min. Three categories of mating activity were recognized (male, female, and simultaneous hermaphrodite); egg-laying activity was also recorded, but courtship was not. For calculation purposes, animals that did not mate or lay eggs during the observation period were assigned a 280-min latency for that activity. Although this approach underestimates the difference in mean latency to mating between strongly positive and control conditions, the effect is relatively small because at least 85% of the animals mated in every experimental condition tested. Statistical significance was assessed by a one-way analysis of variance, followed by Duncan's multiple range test for pairwise comparisons. When time courses of mating activity were compared, statistical significance was assessed by $x^2$ analysis of individual time points. Egg cordon volume was measured at the end of each experiment by ASW displacement in a graduated cylinder and averaged 1.7 ml (1.7 ± 0.2 ml; mean ± S.E.M.). Twenty experiments were performed in this series.

*Results.* Animal-conditioning the ASW with a nonlaying animal resulted in an increase in the percentage of

Handle animal;
inject atrial gland extract

↓

Conditions ASW;
lays eggs

Conditioned ASW
with eggs

Transfer egg layer to
Non-conditioned ASW
without eggs

↓

Add 2 nonlayers

↓

Add 1 nonlayer

↓

2 nonlayers in
conditioned ASW
with eggs

Egg layer, nonlayer in
non-conditioned ASW
without eggs

Handle animal;
no injection

↓

Conditions ASW;
does not lay eggs

Conditioned ASW
without eggs

Transfer nonlayer to
Non-conditioned ASW
without eggs

↓

Add 2 nonlayers

↓

Add 1 nonlayer

↓

2 nonlayers in
conditioned ASW
without eggs

2 nonlayers in
non-conditioned ASW
without eggs

**Figure 6.** Flow diagram of the protocol used in the first series of mating experiments. One animal was injected with an extract of the atrial gland to induce egg laying and placed in a beaker containing aerated non-conditioned ASW; a second animal was handled and placed in a second beaker containing aerated non-conditioned ASW. When egg deposition was complete, the egg layer was transferred to a third beaker and the handled nonlayer to a fourth beaker. Additional nontreated animals were then distributed among the four beakers so that each contained a pair of *Aplysia;* the resulting experimental conditions are indicated at the bottom of the diagram. Mating and egg-laying behaviors were scored at 10-min intervals for 270 min; animals failing to exhibit a behavior during the observation period were assigned a 280-min latency for calculations.

animals mating at early time periods relative to non-conditioned ASW controls (Fig. 7A); the difference was statistically significant at three time periods—40, 50, and 60 min [$x^2(1) > 3.84$ for each; $P < 0.05$]. The mean latency to mating was also reduced (Fig. 7B), but did not differ significantly from that of the non-conditioned ASW controls ($P = 0.29$; one-way analysis of variance). It is important to note that these effects, although small, were consistently observed when the ASW was animal-conditioned. Comparable results were obtained in an independent series of experiments performed in our laboratory (A. R. Gustavson, unpubl. data). The studies used a different pool of *A. brasiliana* and animal-conditioned the ASW for 60 rather than 70 min, but produced quantitatively similar responses. A higher percentage of animals mated at early time periods relative to the non-conditioned ASW controls [$x^2(1) \geq 3.84$ at 30, 40, 70, 80, 90, 100, and 110 min; $P \leq 0.05$ for each]; the mean latency to mating was reduced, but the change was not statistically significant ($P = 0.28$; one-way analysis of variance). The consistency of these two sets of results suggests that animal-derived factors induce mating, but that their activity

or concentration is relatively low under the conditions tested. The idea that animal-derived factors induce mating in *Aplysia* is consistent with a recent report in the literature that the amount of time that *A. fasciata* spend mating is a function of the number of animals available as copulatory partners (Ziv *et al.,* 1989) and thus, presumably, a function of the concentration of animal-derived factors in the ASW.

Similar, but quantitatively larger, effects were observed when the ASW was animal-conditioned by an egg layer and contained an egg cordon (Fig. 7A, B). The percentage of animals mating at early time periods was increased relative to non-conditioned ASW controls, and the difference was statistically significant for every observation period from 10 through 110 min [$x^2(1) \geq 3.84$ for each; $P \leq 0.05$]. The mean latency to mating was significantly reduced ($P = 0.002$; one-way analysis of variance). Assuming that animal-conditioning the ASW with an egg layer is comparable to animal-conditioning with a nonlayer (see below), these results demonstrate that cordon-derived factors induce mating, and suggest that the effects of the animal-derived and cordon-derived factors may be additive.
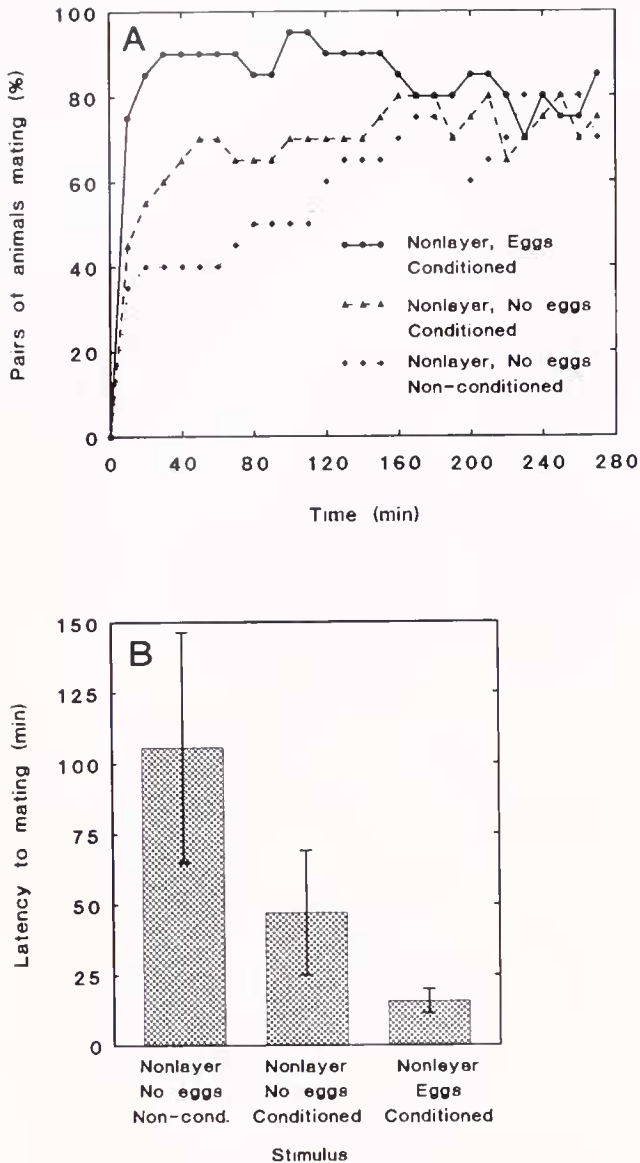
Figure 7. Both animal-derived and cordon-derived factors induce mating activity in *Aplysia brasiliana* (A) The percentage of animals mating at early time periods was increased by animal-conditioning the ASW with a nonlaying animal; the difference was significant at 40, 50, and 60 min [$x^2(1) > 3.84$; $P < 0.05$]. The percentage was further increased by animal-conditioning the ASW with an egg-laying animal and leaving the egg cordon in the ASW; the percentages were significantly higher than those obtained in non-conditioned ASW without an egg cordon at every observation period from 10 through 110 min [$x^2(1) > 3.84$ for each; $P < 0.05$]. The experimental protocol is shown in Figure 6 (n = 20 experiments). Because *Aplysia* tend to mate in bouts lasting approximately 60 min and the bouts are often separated by periods during which no mating occurs (Leonard and Lukowiak, 1987), it is not possible to read a mean latency to mating directly from this graph. (B) The latency to mating (mean ± S.E.M.) was reduced by animal-conditioning the ASW with a nonlaying animal, but the difference was not statistically significant ($P = 0.29$; one-way analysis of variance). The latency was further reduced in animal-conditioned ASW with an egg cordon, and differed significantly from that obtained for non-conditioned ASW without an egg cordon ($P < 0.002$; one-way analysis of variance).

Animals did not distinguish between recent egg layers and nonlayers in non-conditioned ASW without an egg cordon. There were no significant differences in the time courses of mating activity (Fig. 8A), in the latencies to mating (Fig. 8B), or in the sexual role first assumed by the animals (Table I). These results suggest that there is not a prolonged change in the motivational state of the egg layer (*i.e.*, an increase in receptivity to courtship) that persists in the absence of an egg cordon. More importantly, they suggest that the egg cordon, rather than the egg layer, may be primarily responsible for the relatively short latencies to mating observed when animals are actively laying eggs. The experiments did not address the question of whether specific layer-derived or animal-derived factors are required for the induction of mating by egg cordons, however, and this issue was examined in the next series of experiments.

*Induction of mating by egg cordons in non-conditioned ASW: experimental protocol and statistical analyses.* Five animals, selected as described above, were used in each experiment (Fig. 9). One was injected with atrial gland extract and placed in a beaker to lay eggs. When deposition was complete, the egg cordon was removed, quickly rinsed, and transferred to a second beaker containing non-conditioned ASW; a pair of animals was then placed in this beaker and another pair placed in a third beaker that contained only non-conditioned ASW. The resulting experimental conditions are: (1) two nonlayers in non-conditioned ASW with an egg cordon; and (2) two nonlayers in non-conditioned ASW without an egg cordon. Reproductive behavior was assessed for each animal at 10-min intervals for 270 min and analyzed as in the preceding experiments. Egg volume was measured after each experiment and averaged 2.0 ml (2.0 ± 0.3 ml; mean ± S.E.M.). Fifteen experiments were performed.

*Results.* Placing a recently deposited egg cordon in the non-conditioned ASW surrounding two nonlaying animals significantly increased the percentage of animals mating in 9 of the first 14 observation periods [Fig. 10A; $x^2(1) \geq 3.84$ at 20–40 min, 70 min, and 100–140 min; $P \leq 0.05$ for each], and significantly reduced their mean latency to mating relative to the control group (Fig. 10B; $P < 0.01$; one-way analysis of variance). These results demonstrate that cordon-derived factors alone are sufficient to induce mating.

## Induction of egg-laying activity

Egg deposition was also monitored in the experiments described above. Neither animal-derived nor cordon-derived factors significantly affected the percentage of animals laying eggs (Table II), and the low percentages made calculations of mean latency to deposition meaningless. Because *Aplysia brasiliana* lays eggs more frequently when
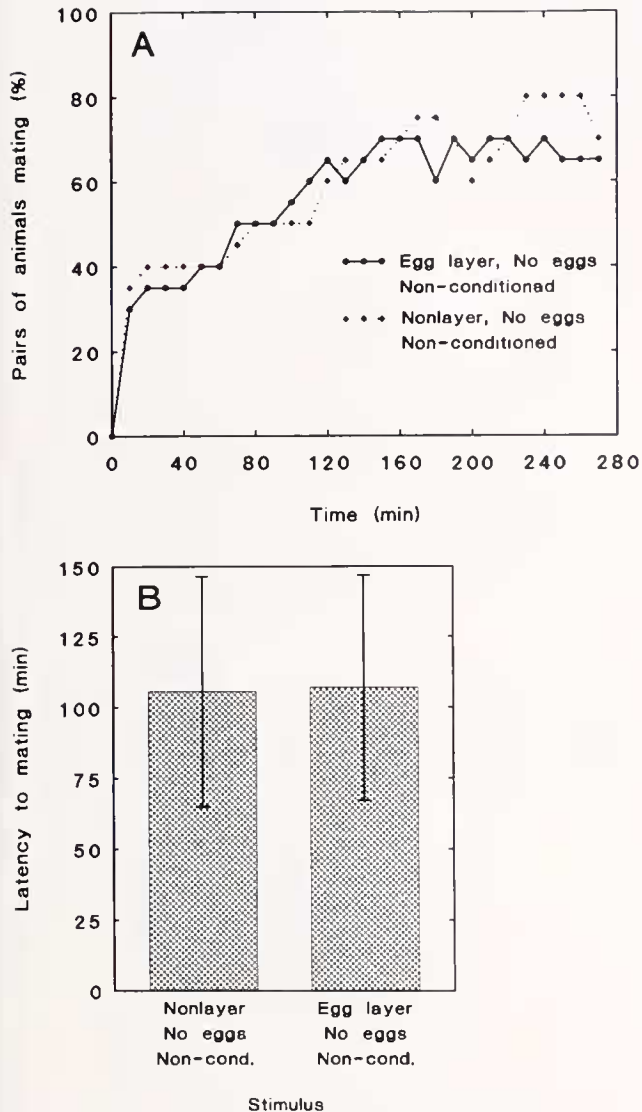
**Figure 8.** *Aplysia brasiliana* does not distinguish between recent egg layers and nonlayers in non-conditioned ASW without an egg cordon. (A) Recent egg layers and nonlayers mated at the same frequency in non-conditioned ASW without an egg cordon. The experimental protocol is shown in Figure 6 (n = 20 experiments). (B) There is no difference in mean latency to mating between recent egg layers and nonlayers in non-conditioned ASW without an egg cordon.

caged alone rather than in pairs (Blankenship *et al.*, 1983), the experiments were repeated with one animal in each beaker rather than two.

*Single-animal experiments: protocol.* Four test animals, selected as described in the mating studies, were used in each experiment. One was placed in each of four beakers and egg-laying activity assessed at 10-min intervals for 270 min. The ASW in each beaker contained a different combination of animal-derived and cordon-derived factors: (1) non-conditioned ASW without an egg cordon (negative control); (2) non-conditioned ASW with an egg

cordon (cordon-derived factors only); (3) animal-conditioned ASW without an egg cordon (animal-derived factors only); and (4) animal-conditioned ASW with an egg cordon (both animal-derived and cordon-derived factors). The conditions in each beaker were established as described in the section on mating (see Figs. 6 and 9). The volumes of the stimulus egg cordons were measured at the end of every experiment and averaged 3.2 ml (3.2 ± 0.6 ml; mean ± S.E.M.). In five experiments, all animals that were not induced to lay eggs were injected with atrial gland extract to verify that they were physiologically competent to do so; all laid egg cordons in response to the injection, demonstrating that the experimental conditions were not interfering with the activity.

*Results.* Neither animal-derived nor cordon-derived factors had a significant effect on egg deposition (Table II).

## Discussion

### Pheromonal attraction

These studies have shown that *Aplysia brasiliana* is not attracted to nonlaying animals in the absence of an egg cordon. The results contrast with those of Lederhendler and colleagues (1977), which showed that *Aplysia dactylomela* is attracted to nonlaying conspecifics and that the magnitude of the attraction increases as the number of stimulus animals increases. We do not know whether the difference reflects species differences or whether it results from differences in experimental design (*e.g.*, from differences in concentration produced by using 5-min rather than 60-min conditioning periods), and we have not examined the possibility that a group of nonlaying *A. brasiliana* would be attractive. We have, however, tested *A. californica* in T-maze experiments and have found that *A. californica*, like *A. brasiliana*, is not attracted to nonlaying conspecifics under these conditions (S. D. Painter, unpubl. data). These results are consistent with earlier studies by Audesirk (1977), which showed that *A. californica* is not attracted to nonlaying animals in Y-maze experiments. Although there is electrophysiological evidence that *A. californica* detects the odors of nonlaying conspecifics (Audesirk and Audesirk, 1977; Chase, 1979), there is no evidence to date that the electrophysiological response is to species-specific odors (Chase, 1979) and no behavioral evidence that the odors are attractive.

*Aplysia brasiliana* is attracted to egg-laying animals with egg cordons. The ability to distinguish egg-laying animals with egg cordons from nonlaying animals without egg cordons was previously described in burrowing studies in this species (Aspey and Blankenship, 1976). In those studies, when a nonlaying animal was introduced into an aquarium containing a burrowed conspecific, the introduced animal burrowed; when an egg-laying animal was

S. D. PAINTER *ET AL.*

**Table I**

*The effects of recent egg deposition on sexual role in* Aplysia brasiliana

| Conditions | Animal | % of animals first mating as | | | % Not mating |
|---|---|---|---|---|---|
| | | Female | Male | Hermaphrodite | |
| Conditioned ASW, egg cordon present[a] | Egg layer | 90 | 10 | 0 | 0 |
| Non-conditioned ASW, no egg cordon[b] | Egg layer | 45 | 30 | 15 | 10 |
| Non-conditioned ASW, no egg cordon[b] | Nonlayer | 40 | 40 | 10 | 10 |

[a] From Painter *et al* (1989). Some egg layers were actively laying eggs when the second animal was introduced into the chamber. The ASW was animal-conditioned by the egg layer. n = 20.

[b] In each case, the experimental animal was introduced into a new chamber at the beginning of the observation period and the ASW was not animal-conditioned. Egg layers were animals that had just completed egg deposition; nonlayers were those that had not laid within the preceding 24 h. The activity pattern of an egg layer in non-conditioned ASW without an egg cordon differed significantly from that of an egg layer in animal-conditioned ASW with an egg cordon [$x^2(3) = 16.67$; $P < 0.005$], but did not differ from that of a nonlayer in non-conditioned ASW without an egg cordon ($x^2 = 1.11$; $0.75 < P < 0.90$). n = 20 for each.

introduced, however, the burrowed animal emerged to mate with it. The distinction is also evident in mating experiments (Painter *et al.*, 1989), which showed that egg-laying animals have significantly shorter latencies to mating than do sexually mature but nonlaying animals. Other species of *Aplysia* also make the distinction. Copulatory

Handle animal;
inject atrial gland extract

↓

Conditions ASW;
lays eggs

Conditioned ASW with egg layer

Transfer eggs to Non-conditioned ASW without eggs

Non-conditioned ASW without eggs

↓

Add 2 nonlayers

↓

Add 2 nonlayers

↓

2 nonlayers in non-conditioned ASW with eggs

↓

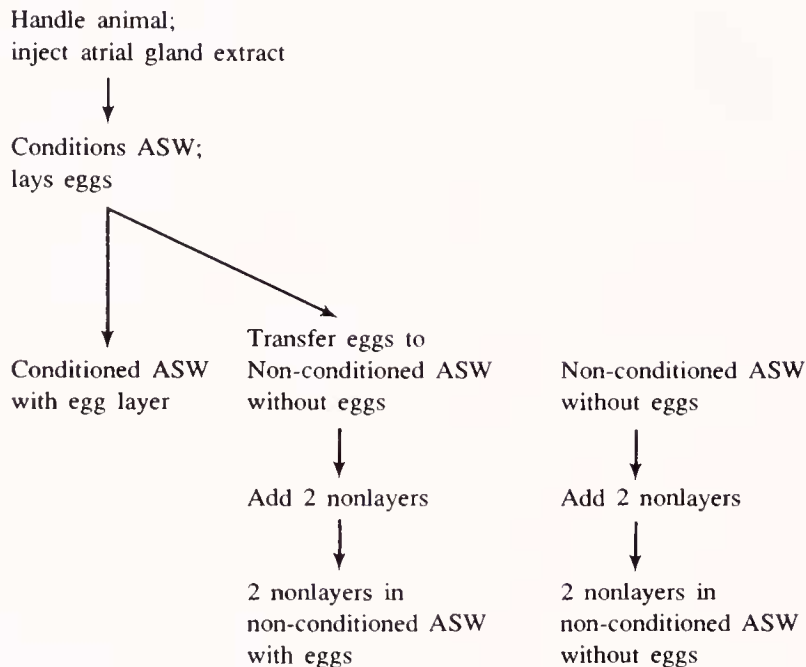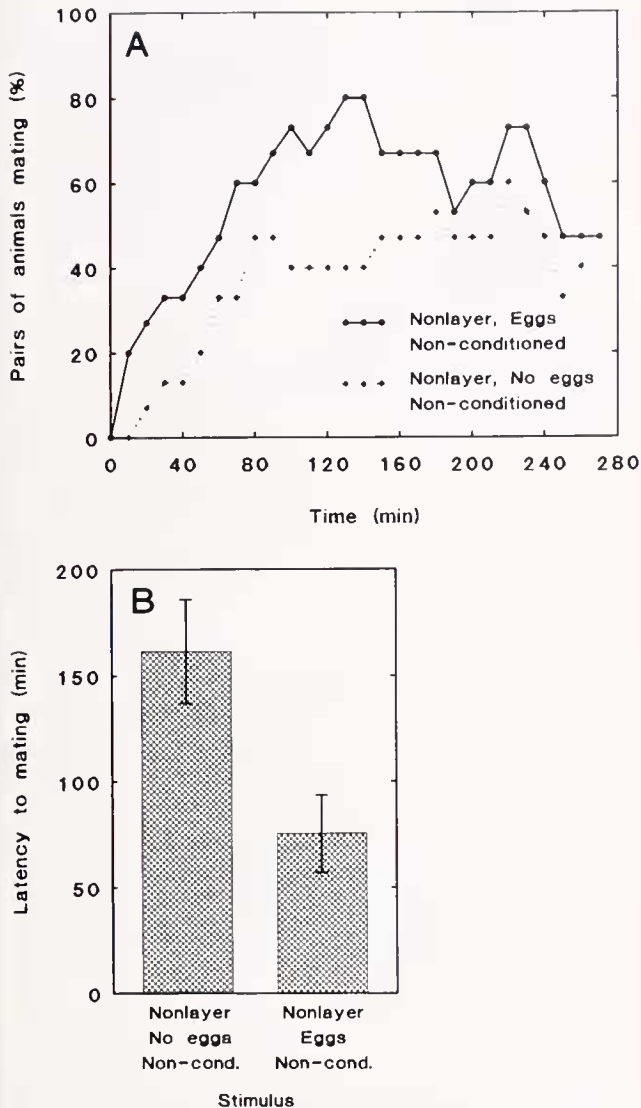2 nonlayers in non-conditioned ASW without eggs

Figure 9.   Flow diagram of the protocol followed in the second series of mating experiments. One animal was injected with an extract of the atrial gland to induce egg laying and placed in a beaker containing aerated non-conditioned ASW. When egg deposition was complete, the egg cordon was removed from the container, quickly rinsed, and transferred to a second beaker containing non-conditioned ASW. Two nontreated animals were then added to the beaker containing the egg cordon, and two others added to a third beaker containing only non-conditioned ASW. The resulting experimental conditions are indicated at the bottom of the diagram. Reproductive behaviors were scored at 10-min intervals for 270 min.

mains to be demonstrated whether the two sets of factors differ biochemically. There is extensive and prolonged contact between the egg layer and its cordon, both during and following oviposition, which would facilitate a transfer of activity between the two.

The attractiveness of a nonlaying animal was increased by placing a recently deposited egg cordon in the same arm of the T-maze, providing the basis for a simple bioassay system in which to identify potential tissue sources of the "cordon-derived" pheromonal activity. Extracts of the *A. californica* atrial gland were assayed in this system and also increased the attractiveness of a nonlaying animal, suggesting that secretory products of the *A. californica* atrial gland (or *A. brasiliana* AG-LE) may contribute to the cordon-derived activity. The extracts each contained 50% of the material in a single atrial gland and were as attractive as *A. brasiliana* egg cordons, suggesting that the *A. brasiliana* and *A. californica* aggregation pheromones may be chemically similar or identical. This issue is currently being examined by HPLC analyses of egg cordon eluates, and by compositional and microsequence analyses of active fractions. It is worth noting that extracts of the *A. californica* atrial gland also induce copulatory activity in *A. brasiliana* (Painter *et al.*, 1989), and that field studies suggest that *Aplysia* aggregation pheromones may not be entirely species-specific (*A. vaccaria*, for example, has been observed in association with *A. californica* aggregations; Kupfermann and Carew, 1974).

### Mating activity

The experiments showed that both animal-derived and cordon-derived factors reduce the latency to mating in

**Figure 10.** High concentrations of animal-derived factors are not required for the induction of mating activity by recently deposited egg cordons. (A) A higher percentage of nonlaying *Aplysia brasiliana* mated at early time periods when an egg cordon was placed in the surrounding non-conditioned ASW; the increase was statistically significant at 20, 30, 40, 70, and 100–140 min [$x^2(1) > 3.84$ in each case; $P < 0.05$]. The experimental protocol is shown in Figure 9 (n = 15 experiments). (B) Placing an egg cordon in the non-conditioned ASW surrounding two nonlaying animals significantly reduced their latency to mating relative to nonlaying animals without an egg cordon ($P < 0.01$; one-way analysis of variance). The experimental protocol is shown in Figure 9 (n = 15 experiments).

chains of *A. californica*, for example, are both more stable and more attractive when the first animal in the chain is laying eggs (Audesirk, 1977).

The present studies have shown that both egg-layer-derived and cordon-derived factors contribute to the attractiveness of an egg-laying animal. Although there are two apparent sources of this pheromonal activity, it re-

**Table II**

*Neither animals nor egg cordons stimulated egg-laying activity in* Aplysia brasiliana

| Conditions | % Animals laying eggs | |
| --- | --- | --- |
| | 2 Animals in chamber | 1 Animal in chamber |
| Non-conditioned ASW | | |
| No egg cordon | 0[a] | 0[b] |
| Egg cordon | 6.7[a] | 6.7[b] |
| Animal-conditioned ASW | | |
| No egg cordon | 7.5[c] | 0[d] |
| Egg cordon | 12.5[c] | 10[d] |

[a] n = 15; 30 possible egg-laying episodes. Not significantly different [$x^2(1) = 2.14$; $0.10 < P < 0.25$].

[b] n = 15; 15 possible egg-laying episodes. Not significantly different [$x^2(1) = 1.07$; $0.25 < P < 0.50$].

[c] n = 20; 40 possible egg-laying episodes. Not significantly different [$x^2(1) = 1.44$; $0.10 < P < 0.25$].

[d] n = 20; 20 possible egg-laying episodes. Not significantly different [$x^2(1) = 2.22$; $0.10 < P < 0.25$].

*Aplysia*, and suggest that their effects are additive. We did not record courtship behavior in any of these experiments, however, and do not know whether the effect results from a reduction in the latency to courtship (*i.e.*, an increase in male behaviors), a reduction in the duration of courtship (*i.e.*, an increase in female receptivity), or from some combination of the two.

The experiments also showed that the active animal-derived factors are not uniquely associated with the egg layer. Although the effect was small, animal-conditioning the ASW with a handled nonlayer consistently reduced the latency to mating, and animals did not distinguish between recent egg layers and handled nonlayers in non-conditioned ASW without an egg cordon. These results suggest that cordon-derived factors are primarily responsible for the relatively short latencies to mating observed with egg-laying animals.

Although previous studies reported that 90% of egg-laying animals initially mated as females (Painter *et al.*, 1989), only 45% did so in the present studies. The difference probably reflects differences in experimental protocol, two of which are particularly significant. First, the animals in the previous studies were tested in animal-conditioned ASW with an egg cordon rather than in non-conditioned ASW without an egg cordon. This suggests that the increase in receptivity might have been due to pheromonal factors rather than a persistent change in motivational state caused by bag-cell activity, ovulation, or movement of eggs through the reproductive tract. It is worth noting that bag-cell activity normally precedes ovulation *in vivo* (*A. brasiliana*, Dudek *et al.*, 1977), that bag-cell products affect neuronal activity (*A. californica*, Mayeri, 1979; Stuart and Strumwasser, 1980), and that certain aspects of egg-laying behavior depend upon movement of eggs through the reproductive tract (*A. brasiliana*, Cobbs and Pinsker, 1982). Second, the time period between injecting atrial gland extract and adding the second animal was held constant at 60 min in the previous studies (Painter *et al.*, 1989), but varied depending upon the time required to complete egg deposition in the present studies, averaging approximately 70 min. Although the egg cordons were smaller in the first study (0.8 compared to 1.7 ml), it is likely that some proportion of the animals were laying eggs when the second animal was introduced. Because egg-laying animals cannot simultaneously mate as males (Leonard and Lukowiak, 1987), these animals would skew the percentages toward mating as a female. In either case, the experiments suggest that, if the receptivity of the egg layer is increased by egg deposition, the effect is dependent on cordon-derived pheromones.

## Egg-laying activity

Although both cordon-derived and animal-derived factors attracted animals and induced mating in our stud-ies, they did not elicit egg deposition. Comparable results were obtained with either one or two animals in the test chamber, demonstrating that egg deposition was not being influenced to a significant degree by the accessibility of a potential mate. It is more likely that the lack of effect resulted from the small percentage of animals that are in the correct motivational state to respond to pheromonal stimulation. This suggestion is based on a comparison with earlier studies by Audesirk (1977), which indicated that egg-laying *Aplysia californica* induce egg laying in conspecifics. In contrast to the present studies, however, the *A. californica* experiments were designed to detect low-frequency events. An egg-laying animal was placed in one of two aquaria, each of which contained four to five individually caged animals, and activity was monitored until an animal in one of the two aquaria began to lay eggs; in 12 of the 15 trials, the first animal to lay eggs was in the aquarium containing the egg-laying animal stimulus, and egg deposition usually began within 3 h. Although the two series of experiments are not identical in design, egg-laying activity on a per-animal basis over a 3- to 3.5-h period is similar: 4–5% laid eggs in the negative control aquarium and 16–20% in the aquarium containing the egg-laying animal stimulus (Audesirk, 1977), compared to 0% in our negative control (non-conditioned ASW without an egg cordon) and 10–12.5% in the animal-conditioned ASW with an egg cordon. The low percentage of animals responding to this stimulus may have physiological significance, allowing some animals to mate as males and providing a mechanism by which the aggregation can be maintained over a relatively long period of time (see Audesirk, 1977).

## A comparison of aggregation and mating pheromones

These studies demonstrate that both animal-derived and cordon-derived factors contribute to the development and maintenance of breeding aggregations, by attracting animals to the area and inducing them to mate. Animal-derived and cordon-derived factors appear to be equally attractive. There were no significant differences in levels of attraction or patterns of responses to: (1) an egg layer without eggs; (2) an egg layer with eggs; (3) a nonlayer with eggs; and (4) eggs without any animal. The animal-derived pheromones appear to be uniquely associated with the egg layer since nonlaying animals without egg cordons were not attractive.

Cordon-derived factors appear to be primarily responsible for the induction of mating by an animal that is actively laying eggs. This idea is supported by three sets of observations. First, animals do not distinguish between recent egg layers and nonlayers in non-conditioned ASW without an egg cordon; there were no significant differences in the time courses of mating activity or in the mean

latencies to mating in our studies. Animals do distinguish between recent egg layers with egg cordons and nonlayers without egg cordons (Aspey and Blankenship, 1976; Painter *et al.*, 1989), however, suggesting that the distinction is based on cordon-derived, rather than animal-derived, factors. Second, placing a recently deposited egg cordon in the non-conditioned ASW surrounding two nonlaying animals significantly reduced their mean latency to mating relative to non-conditioned ASW controls. Animal-conditioning the ASW also reduced the mean latency to mating relative to the controls but, in this case, the reduction was not statistically significant. Thus, although cordon-derived and animal-derived factors are each sufficient to induce mating, the cordon-derived factors are likely to play the more significant role in eliciting the activity. Finally, the animal-derived factors that induce mating are associated with both egg layers and nonlayers, suggesting that they are not responsible for the differences in mating activity observed in the two groups (Painter *et al.*, 1989).

From a behavioral point of view, it is somewhat puzzling that an egg layer is more attractive than a nonlayer in nonconditioned ASW without an egg cordon, but does not have a shorter latency to mating. It should be kept in mind, however, that these conditions rarely, if ever, occur in the natural environment. Egg-laying animals maintain contact with their egg cordons for extended periods of time, and often begin mating before deposition is complete (*A. brasiliana*, Blankenship *et al.*, 1983).

*Cordon-derived pheromones and the atrial gland*

It has yet to be determined whether the cordon-derived factors that induce aggregation are identical to the ones that induce mating. However, it is important to note that the effects of recently deposited egg cordons can be quantitatively mimicked in both assay systems by placing an extract of the atrial gland into the surrounding ASW (aggregation, present studies; mating, Painter *et al.*, 1989); extracts containing approximately 50% of the material in a single gland (T-maze experiments; 67% was tested in mating experiments) are as active as one egg cordon, suggesting that these effects may be of physiological rather than purely pharmacological interest.

It is worth noting that the atrial gland is one of only five exocrine organs associated with the female reproductive tract, and that it is the last of the five to contact the egg cordon before deposition (*A. californica*, Coggeshall, 1972; Painter *et al.*, 1985). Moreover, the gland synthesizes large amounts of a few structurally related precursor proteins and processes them into small families of structurally related peptides with similar pharmacological properties. It stores milligram quantities of the products (*A. californica*, Heller *et al.*, 1980; Nagle *et al.*, 1986, 1988b; Roth-

man *et al.*, 1986), and releases them in response to depolarizing stimuli (*A. californica*, Arch *et al.*, 1980). These characteristics are consistent with the atrial gland being a tissue source of the "cordon-derived" pheromonal activity, but it remains to be determined whether the atrial gland serves this function in the animal and whether the "cordon-derived" pheromones are products of the ELH-related genes expressed in the gland.

## Literature Cited

**Arch, S., J. Lupatkin, T. Smock, and M. Beard. 1980.** Evidence for an exocrine function of the *Aplysia* atrial gland. *J. Comp. Physiol.* **151:** 131–137.

**Arch, S., T. Smock, R. Gurvis, and C. McCarthy. 1978.** Atrial gland induction of the egg-laying response in *Aplysia californica J. Comp. Physiol.* **128:** 67–70.

**Aspey, W. P., and J. E. Blankenship. 1976.** *Aplysia* behavioral biology. I. Induced burrowing in swimming *A. brasiliana* by a burrowed conspecific. *Behav. Biol.* **17:** 301–312.

**Audesirk, T. E. 1977.** Chemoreception in *Aplysia californica*. III. Evidence for pheromones influencing reproductive behavior. *Behav. Biol.* **20:** 235–243.

**Audesirk, T. E. 1979.** A field study of growth and reproduction in *Aplysia californica*. *Biol. Bull.* **157:** 407–421.

**Audesirk, T. E., and G. J. Audesirk. 1977.** Chemoreception in *Aplysia californica*. II. Electrophysiological evidence for detection of the odors of conspecifics. *Comp. Biochem. Physiol., pt. A* **56:** 267–270.

**Beard, M., L. Millechia, C. Masuoka, and S. Arch. 1982.** Ultrastructure of secretion in the atrial gland of a mollusc *(Aplysia)*. *Tissue and Cell* **14:** 297–308.

**Blankenship, J. E., and R. E. Coggeshall. 1976.** The abdominal ganglion of *Aplysia brasiliana*: a comparative morphological and electrophysiological study, with notes on *A. dactylomela*. *J. Neurobiol.* **7:** 383–405.

**Blankenship, J. E., M. K. Rock, L. C. Robbins, C. A. Livingston, and H. K. Lehman. 1983.** Aspects of copulatory behavior and peptide control of egg laying in *Aplysia*. *Fed. Proc.* **42:** 96–100.

**Chase, R. 1979.** An electrophysiological search for pheromones of *Aplysia californica*. *Can. J. Zool.* **57:** 781–784.

**Chiu, A. Y., M. W. Hunkapiller, E. Heller, D. K. Stuart, L. E. Hood, and F. Strumwasser. 1979.** Purification and primary structure of the neuropeptide egg-laying hormone of *Aplysia californica*. *Proc. Natl. Acad. Sci. USA* **76:** 6656–6660.

**Cobbs, J. S., and H. M. Pinsker. 1982.** Role of bag cells in egg deposition of *Aplysia brasiliana* II. Contribution of egg movement to elicited behaviors. *J. Comp. Physiol., pt. A* **147:** 537–546.

Coggeshall, R. E. 1972.    The structure of the accessory genital mass in *Aplysia californica. Tissue and Cell* 4: 105–127.

Dudek, F. E., and J. E. Blankenship. 1977.    Neuroendocrine cells of *Aplysia brasiliana.* 1. Bag cell potentials and afterdischarge. *J. Neurophysiol.* 40: 1301–1311.

Dudek, F. E., J. S. Cobbs, and H. M. Pinsker. 1979.    Bag cell electrical activity underlying spontaneous egg laying in freely behaving *Aplysia brasiliana. J. Neurophysiol.* 42: 804–817.

Frazier, W. T., E. R. Kandel, I. Kupfermann, R. Waziri, and R. E. Coggeshall. 1967.    Morphological and functional properties of identified neurons in the abdominal ganglion of *Aplysia californica. J. Neurophysiol.* 30: 1288–1351.

Heller, E., L. K. Kaczmarek, M. W. Hunkapiller, L. E. Hood, and F. Strumwasser. 1980.    Purification and primary structure of two neuroactive peptides that cause bag cell afterdischarge and egg-laying in *Aplysia. Proc. Natl. Acad. Sci. USA* 77: 2328–2332.

Jahan-Parwar, B. 1976.    Aggregation pheromone from the egg-mass of *Aplysia. The Physiologist* 19: 240.

Kupfermann, I., and T. Carew. 1974.    Behavior patterns of *Aplysia californica* in its natural environment. *Behav. Biol.* 12: 317–337.

Kupfermann, I., and E. R. Kandel. 1970.    Electrophysiological properties and functional interconnections in two symmetrical neurosecretory clusters (bag cells) in abdominal ganglion of *Aplysia. J. Neurophysiol.* 33: 865–876.

Lederhendler, I. I., K. Herriges, and E. Tobach. 1977.    Taxis in *Aplysia dactylomela* (Rang, 1828) to water-borne stimuli from conspecifics. *Anim. Learning Behav.* 5: 355–358.

Leonard, J. L., and K. Lukowiak. 1987.    The behavior of *Aplysia californica* Cooper (Gastropoda: Opisthobranchia). *Behavior* 98: 320–360.

MacGinitie, G. E. 1934.    The egg-laying activities of the sea hare *Tethys californica* (Cooper). *Biol. Bull.* 62: 300–303.

Mahon, A. C., J. R. Nambu, R. Taussig, M. Shyamala, A. Roach, and R. Scheller. 1985.    Structure and expression of the egg-laying hormone gene family in *Aplysia. J. Neurosci.* 5: 1872–1880.

Mayeri, E. 1979.    Local hormonal modulation of neural activity in *Aplysia. Fed. Proc.* 38: 2103–2108.

Nagle, G. T., S. D. Painter, J. E. Blankenship, J. D. Dixon, and A. Kurosky. 1986.    Evidence for the expression of three genes encoding homologous atrial gland peptides that cause egg laying in *Aplysia. J. Biol. Chem.* 261: 7853–7859.

Nagle, G. T., S. D. Painter, J. E. Blankenship, J. V. A. Choate, and A. Kurosky. 1988a.    The bag cell egg-laying hormones of *Aplysia brasiliana* and *Aplysia californica* are identical. *Peptides* 9: 867–872.

Nagle, G. T., S. D. Painter, J. E. Blankenship, and A. Kurosky. 1988b.    Proteolytic processing of egg-laying hormone-related precursors in *Aplysia.* identification of peptide regions critical for biological activity. *J. Biol. Chem.* 263: 9223–9237.

Nambu, J. R., and R. H. Scheller. 1986.    Egg-laying hormone genes of *Aplysia:* evolution of the ELH gene family. *J. Neurosci.* 6: 2026–2036.

Painter, S. D., A. R. Gustavson, V. K. Kalman, G. T. Nagle, and J. E. Blankenship. 1989.    Induction of copulatory behavior in *Aplysia:* atrial gland factors mimic the excitatory effects of freshly deposited egg cordons. *Behav. Neural Biol.* 51: 222–236.

Painter, S. D., V. K. Kalman, G. T. Nagle, R. A. Zuckerman, and J. E. Blankenship. 1985.    The anatomy and functional morphology of the large hermaphroditic duct of three species of *Aplysia,* with special reference to the atrial gland. *J. Morphol.* 186: 167–194.

Pinsker, H. M., and D. W. Parsons. 1985.    Temperature dependence of egg laying in *Aplysia. J. Comp. Physiol., pt. B* 56: 21–27.

Rothman, B. S., D. H. Hawke, R. O. Brown, T. D. Lee, A. A. Dehghen, J. E. Shively, and E. Mayeri. 1986.    Isolation and primary structure of the califins, three biologically active ELH-like peptides from the atrial gland of *Aplysia. J. Biol. Chem.* 261: 1616–1623.

Rothman, B. S., E. Mayeri, R. O. Brown, P.-M. Yuan, and J. E. Shively. 1983.    Primary structure and neuronal effects of α-bag cell peptide, a second candidate neurotransmitter encoded by a single gene in bag cell neurons of *Aplysia. Proc. Natl. Acad. Sci. USA* 80: 5753–5757.

Scheller, R. H., J. F. Jackson, L. B. McAllister, B. S. Rothman, E. Mayeri, and R. Axel. 1983.    A single gene encodes multiple neuropeptides mediating a stereotyped behavior. *Cell* 32: 7–22.

Stuart, D. K., A. Y. Chiu, and F. Strumwasser. 1980.    Neurosecretion of egg-laying hormone and other peptides from electrically active bag cell neurons of *Aplysia. J. Neurophysiol* 43: 488–498.

Stuart, D. K., and F. E. Strumwasser. 1980.    Neuronal sites of action of a neurosecretory peptide, egg-laying hormone, in *Aplysia californica. J. Neurophysiol.* 43: 499–519.

Susswein, A. J., and M. Benny. 1985.    Sexual behavior in *Aplysia fasciata* induced by homogenates of the large hermaphroditic duct. *Neurosci. Lett.* 59: 325–330.

Susswein, A. J., S. Gev, Y. Achituv, and S. Markovitch. 1984.    Behavioral patterns of *Aplysia fasciata* along the Mediterranean coast of Israel. *Behav. Neural Biol.* 41: 7–22.

Susswein, A. J., S. Gev, E. Feldman, and S. Markovitch. 1983.    Activity patterns and time budgeting of *Aplysia fasciata* under field and laboratory conditions. *Behav. Neural Biol.* 39: 203–220.

Switzer-Dunlap, M., and M. G. Hadfield. 1977.    Observations on development, larval growth and metamorphosis of four species of Aplysiidae (Gastropoda: Opisthobranchia) in laboratory culture. *J. Exp. Mar. Biol. Ecol.* 29: 245–261.

Thompson, T. E., and A. Bebbington. 1969.    Structure and function of the reproductive organs of three species of *Aplysia* (Gastropoda: Opisthobranchia). *Malacologia* 7: 347–380.

Ziv, I., M. Benni, S. Markovich, and A. Susswein. 1989.    Motivational control of sexual behavior in *Aplysia fasciata:* sequencing and modulation by sexual deprivation and by addition of partners. *Behav. Neural Biol.* 52: 180–193.